STUDIES ON THE BIOCHEMISTRY OF TETRAHYMENA. VII. RIBOFLAVIN, PANTOTHEN, BIOTIN, NIACIN AND PYRIDOXINE IN THE GROWTH OF T. GELEII W

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With the substitution of chemically known materials for all but one fraction in the medium for the growth of Tetrahymena it has been possible to determine with some degree of exactness the specific vitamin requirements of this important ciliate. When proteins, such as casein or gelatin, or peptones are used as the base medium it has been impossible to determine the importance of those vitamins which were stable to treatments which would not also destroy other essential materials. Using these types of media, claims have been made for the essential nature of thiamine and of riboflavin for *Tetrahymena geleii* (Hall and Cosgrove, 1944; Hall, 1944). It was earlier indicated (Kidder and Dewey, 1942) and later conclusively proven (Kidder and Dewey, 1944; 1945a; 1945b) that at least eight strains of Tetrahymena could grow in a medium in which the thiamine had been destroyed.

When it was found that *T. geleii* could be grown successfully in a mixture of amino acids (Kidder and Dewey, 1945c) and that two of the three "unknown growth factors" could be replaced with nucleic acid derivatives (Kidder and Dewey, 1945d) and that the remaining "unknown growth factor" (Factor II) was relatively stable and was not adsorbed readily on activated charcoal, it became possible to examine the effects of the omission of a number of the vitamins. Hitherto these vitamins had been added routinely to guard against the possibility of any one of them proving to be a limiting factor. It was found (Kidder, 1945) that folic acid is an essential growth factor for *T. geleii* W, this fact being obscured previously by the necessary inclusion of Factor I (containing folic acid) as the lead acetate precipitate fractions of raw materials, the Factor I activity being readily absorbable on activated charcoal.

The present work has been made possible by the utilization of a number of different treatments of the Factor II preparations and the inclusion of all other constituents of the medium as chemically pure materials. Furthermore, this work would not have been possible without the employment of a microbiological method for the detection of traces of the growth factors under consideration. We have utilized *Lactobacillus casei* as a tool in this study, and while we have made no attempts to assay various preparations quantitatively, we have used the bacterial method for determining the total lack of the vitamin under immediate consideration. It has been possible also, to show that the ciliate possesses the ability to synthesize certain of the B vitamins, by determining the increase of the vitamin by the *L. casei* test after the growth of the ciliate.

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Materials and Methods

Organisms

The ciliate used in this study was *Tetrahymena geleii* W, which has been maintained in pure (bacteria-free) culture in this laboratory for a number of years and which has been used in numerous previous studies (Kidder and Dewey, 1942–1945). The organism has been grown in amino acid media for the past one and one-half years and all inocula for the present series were taken from these stocks.

Lactobacillus casei 912 was used for the microbiological testing of experimental media. This organism was obtained from the Squibb Institute for Medical Research through the courtesy of Dr. Vincent Groupé. Stocks were carried in yeast extract-dextrose-agar stab cultures, transplants being made at monthly intervals, incubated at 37° C. for 24 hours and then placed in the refrigerator.

Ciliate base medium

One type of base medium was used routinely. This appears in Table I with the complete supplements. Each vitamin under investigation was omitted from the medium separately.

Preparation of Factor II

It has been necessary to treat the Factor II preparations in various appropriate ways in order to eliminate the different vitamins studied. In the earlier work (Kidder and Dewey, 1945d) the prime consideration in the Factor II preparation was the elimination of Factors I and III activity, and the methods used did not necessarily render the preparation vitamin free. In this study the inclusion of Factor I and Factor III activity was of no particular importance, and so attempts were made to eliminate the vitamin under consideration and still retain maximum Factor II activity. This latter was not always possible as some of the treatments used not only removed or destroyed the vitamin but also lowered the Factor II activity. Nevertheless preparations which were satisfactory for this study were obtained, and these will be described under the heading of each vitamin.

Riboflavin-free preparation (8L531).

Liver Fraction L [2] (50 grams) was dissolved in one liter of distilled water and a 40 per cent solution of normal lead acetate was added until no more precipitate formed. The precipitate was removed by filtration with the aid of Celite and discarded. The filtrate was neutralized with NaOH and treated with an excess of basic lead acetate. The second precipitate was removed and discarded, the excess lead removed with 9 per cent oxalic acid and the excess oxalic removed as the oxalate with $Ca(OH)_2$. Tests at this stage showed the presence of large amounts of riboflavin, but after adsorption with 10 grams of Norit A for one hour at room temperature at pH 3.5 the riboflavin had been quantitatively removed. This preparation was used in a concentration of one part in ten parts of final medium.

[2] Furnished through the courtesy of Dr. David Klein and the Wilson Laboratories.

TABLE I

Base Medium

	mg./ml		micrograms/ml.
l(+)-arginine mono-hydrochloride	0.82	biotin methyl ester[3]	0.00005
l(−)-histidine mono-hydrochloride	0.10	calcium pantothenate[3]	0.10
dl-isoleucine	0.35	thiamine hydrochloride	0.10
dl-leucine	0.35	nicotinamide[3]	0.10
dl-lysine	0.60	riboflavin[3]	0.10
dl-methionine	0.34	pyridoxine hydrochloride[3]	0.10
dl-phenylalanine	0.14	*p*-aminobenzoic acid	0.10
dl-serine	0.04	*i*-inositol	1.00
dl-threonine	0.20	choline chloride	1.00
l(−)-tryptophane	0.10	folic acid concentrate[4]	1.00
dl-valine	0.20		
dextrose	2.00		mg./ml.
$MgSO_4.7H_2O$	0.10	hydrolyzed yeast nucleic acid[5]	0.05
K_2HPO_4	0.10		
$CaCl_2.2\ H_2O$	0.05	Factor II preparation (see text)	
$FeCl_3.6\ H_2O$	0.00125		
$MnCl_2.4\ H_2O$	0.00005		
$ZnCl_2$	0.00005		

Pantothen-free preparation (8L531H).

Although pantothenic acid is adsorbed on activated charcoal the time and temperature allowed in preparing the riboflavin-free medium is insufficient for the complete removal of pantothen. Raising the temperature, increasing the time, or increasing the amount of Norit used was not practical as the Factor II activity was greatly reduced (Kidder and Dewey, 1945d). Therefore advantage was taken of the sensitivity of pantothenic acid to alkali and heat and the riboflavin-free preparation was adjusted to pH 10.0 with NaOH and autoclaved for two hours. The Factor II activity was somewhat reduced by this treatment, but the preparation was entirely satisfactory for use. *L. casei* tests showed that the pantothenic acid content had been lowered to an insignificant amount. This preparation was used in concentrations of one part in ten parts of final medium.

Biotin-free preparation (8L5C1)

The most active biotin-free preparation, and therefore the one used in this study, was made in the following manner. Ten grams of Liver Fraction L was dissolved in 200 ml. of distilled water and brought to boiling. To this boiling mixture were added 10 ml. of a 10 per cent solution of $NaHSO_3$ and 10 ml. of a 10 per cent solution of $CuSO_4$, and boiling was continued for 3–5 minutes. The precipitate was removed on a fluted filter and the process repeated once. The copper was removed as CuS after treating with 15 per cent Na_2S and the sulfate and sulfite removed as the barium salts after treatment with $Ba(OH)_2$. The volume of the filtrate was reduced to 200 ml. and a 100 ml. aliquot was adjusted to pH 3.5. Two grams

[3] Omitted singly in the appropriate series of experiments.

[4] The folic acid concentrate used had a potency of 5000 and was furnished through the courtesy of Dr. R. J. Williams.

[5] Assays of the hydrolyzed yeast nucleic acid with *L. casei* showed it to be free of riboflavin, pantothen, biotin, niacin, and pyridoxine but appreciable amounts of folic acid were present.

of Norit A was added and adsorption allowed to continue for one hour at room temperature with constant stirring. This preparation was used in a concentration of one part in twenty parts of final medium.

Niacin-free preparation (8L5C2)

The use of copper precipitation, described above, was designed for the removal of nicotinic acid. While most of the niacin activity was removed by this method, as shown by the *L. casei* test, enough remained to warrant further treatment. Accordingly the filtrate from the copper precipitation was extracted with *n*-butanol for 96 hours in a continuous extraction apparatus (Wilson, Grauer, and Saier, 1940). It is known that nicotinamide is readily extracted with butanol, and after this treatment the extract was found to be entirely devoid of niacin activity, even when tested with *L. casei* in amounts four times greater than those used as a supplement for the ciliate. This preparation was used in a concentration of one part in twenty parts of final medium.

Pyridoxine-free preparation (8L531L)

This preparation was the least successful of any used. While it was possible to treat crude extracts and various filtrates in ways which would remove all pyridoxine activity for *L. casei,* it was usually found that the Factor II activity was also lowered to a point where the preparation was very inferior as a ciliate supplement. Therefore, the most satisfactory preparation, and the one finally used, was very low in Factor II activity, and the results obtained cannot be compared directly with those of the other vitamins tested. This preparation was made by exposing an alkaline lead acetate filtrate fraction (8L531), to direct illumination from a 300 watt electric bulb at a distance of 8 inches for a period of 72 hours. This method was used by Hochberg et al (1943) for pyridoxine destruction. Besides the destruction of appreciable amounts of the Factor II, another disadvantage of the technique was the excessive evaporation which took place during the treatment. It was necessary to add distilled water at frequent intervals to prevent the preparation from drying down. This preparation was used in a concentration of one part in ten parts of final medium.

Assay procedure

The base medium employed for the testing of the various preparations was the 16 amino acid mixture suggested by Hutchings and Peterson (1943). This was chosen in preference to the casein hydrolysate medium of Landy and Dicken (1942) because of the known composition of the former and the fact that lower blanks can be obtained. While the amino acid medium does not permit the production of as much acid by the bacteria it is very satisfactory for determining the presence or absence of a known vitamin.

Because of the scarcity of amino acids we have modified the usual procedure. The amino acid medium is made up for stock in double strength and the sugar, acetate, salts, purines and pyrimidine are added in double strength. For a test, this complete base medium is measured into 125 × 7 mm. Pyrex tubes in one ml. volumes. The material to be tested is added in appropriate amounts and a mixture

of the vitamins, minus the one for which the preparation is being tested, is added. The volume is then made up to 2 ml. with distilled water. Two controls were run with each test, one containing base medium and a complete set of supplements except for the vitamin under test. The second control contained the base medium plus the complete supplement and plus the Factor II preparation. The first served as a control on carry-over growth. The second was a control on the possible toxicity of the Factor II preparation. When titrations were made the figure from the first control was subtracted from the figure from the unknown preparation. Inasmuch as a small volume of medium was used it was found advantageous and more accurate to reduce the standard hydroxide to 0.05 N. The NaOH was standardized with 0.05 N oxalic acid, and the amount of acid produced after 96 hours of growth at 37° C. was titrated directly, using brom thymol blue as an indicator. The longer incubation period was used for maximum acid production, for in this way the test becomes more sensitive for traces of vitamins.

After many trials, the usual drop method of inoculation of *L. casei* was abandoned in favor of inoculating with a straight needle. This eliminates the necessity for washing the bacteria and blanks are just as low. The inocula were always made from yeast extract cultures which had incubated for 18–24 hours at 37° C.

While standard curves, using this method, have been made for all the vitamins studied the results obtained with our preparations do not permit quantitative statements as to amounts inasmuch as the tests were always made at very high levels and stimulatory materials in the Factor II preparations were invariably present. We were interested, moreover, first in the determination of the vitamin-free condition of our media, and second, in the biosynthesis of the vitamins by the ciliates. In the latter case, assays were employed on the medium before and after ciliate growth and the difference of acid production between the two compared directly.

It has been pointed out (see Cheldelin et al, 1942) that many of the B vitamins occur in a bound form in tissues and must be liberated by some means for satisfactory tests. There was the possibility that bound vitamins in the Factor II preparations might be available for the ciliate but not for *L. casei*, and these would invalidate any conclusions which were based on the vitamin-free nature of the preparation by the *L. casei* test. Enzymatic digestion was carried out on all the preparations, therefore, in order to test for the total vitamin content. Accordingly takadiastase and pepsin in quantities of one per cent each of the total solids of the preparation to be tested were used. The preparation was allowed to digest under toluene for 24 hours at 37° C. at pH 3.5. After steaming, the digest was added to the assay tubes, as described above, and a control of equivalent amounts of the enzymes added to parallel tubes. This latter control is obviously necessary as the enzymes are not vitamin-free. Data on the results of assays of the Factor II preparations used are presented in Table II.

Ciliate cultures

It was the usual practice, when testing for the effects of one of the known vitamins, to grow the ciliate through at least three serial tube transplants in the medium containing the vitamin being investigated, paralleled with the same medium minus the vitamin. Transplants were made at 72 hour intervals with a bacteriological loop delivering approximately 0.005 ml. of fluid. All incubation was at

25° C. Growth rate was followed by inoculating appropriate amounts of third transplant ciliates (36 hours old) into like media in culture flasks (Kidder, 1941). After inoculation of the flasks (as near 100/ml. as possible) samples were drawn and the initial inoculum determined. Growth thereafter was ascertained by sampling at intervals until the termination of the experiment. In all cases the flask series were repeated at least once and the figures averaged.

TABLE II

Assay of Factor II Preparations with Lactobacillus casei 912

No.	Additions	Vitamin omitted from base medium					
		Riboflavin	Pantothen	Biotin	Nicotinamide	Pyridoxine	Folic acid
1	None	0.05	0.00	0.05	0.49	0.12	0.62
2	Enzyme preparation	0.13	0.38	0.17	0.75	0.10	0.90
3	8L531	0.05	3.79	2.88	4.72	3.86	0.00
4	8L531H	0.03	0.12	2.79	4.80	2.60	0.00
5	8L5C1	0.00	1.53	0.07	0.22	3.10	4.36
6	8L5C2	3.42	0.87	2.93	0.00	3.65	4.65
7	8L531L	0.00	3.74	3.00	4.51	0.09	0.00

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks. Line 2 corrected for carry-over growth (Line 1). Lines 3–7 corrected for vitamin content of enzyme preparation (Line 2).

One obvious objection to the flask technique is the possibility of introducing the vitamin being investigated from the rubber vaccine tip used in the sampling port. This possibility was diminished by boiling the vaccine tips for one hour previous to setting up the flasks. As a check on the tips uninoculated flasks were manipulated in the same manner as the experimental cultures and the samples tested with *L. casei* for the vitamin being studied. In no case were these detectable amounts of the vitamins present. Sampling needles were made chemically clean as well as sterile before use.

Growth rate during the logarithmic phase was calculated by the formula $g = \frac{t \log 2}{\log b - \log a}$ where t = the time in hours during which the population has been increasing exponentially, a =the number of cells per unit volume at the beginning, and b = the number of cells at the end of time, t.

RESULTS

After obtaining Factor II preparations which were free of the vitamins to be studied, preliminary experiments were set up to determine which vitamins, if any, were essential growth factors for *Tetrahymena geleii* W. Accordingly serial transplants were made in the appropriate media, one set with the vitamin present, and the other with the vitamin omitted. It was immediately apparent that the ciliate lacked all ability to synthesize folic acid (Kidder, 1945) but the absence of none of the other vitamins did more than lower the growth rate and the yield. Growth in

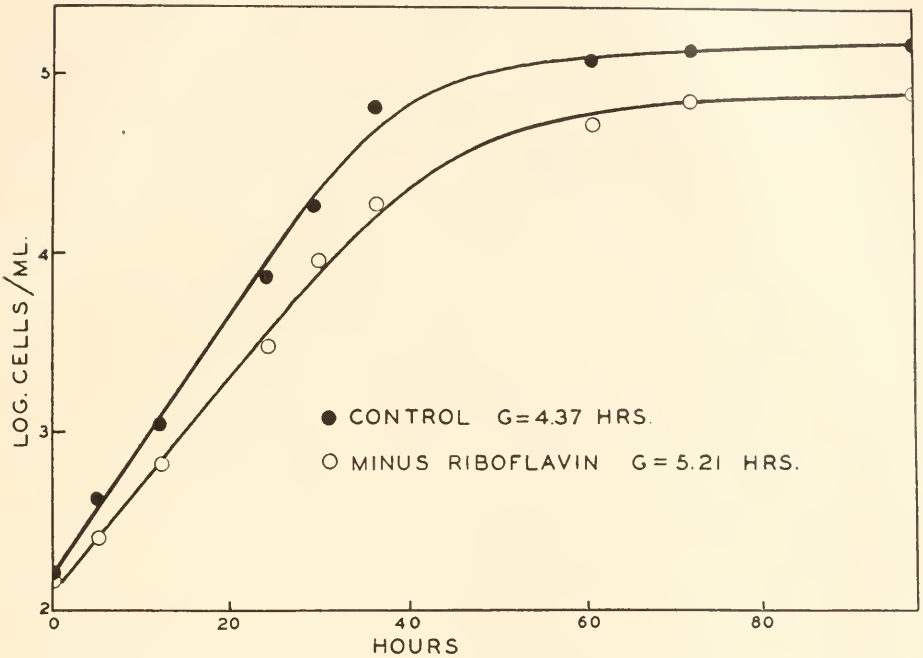

FIGURE 1. Effect of the omission of riboflavin. Factor II preparation used was 8L531. Average of two separate experiments.

TABLE III

Summary of Growth Data

Medium	Generation time in hours	Population per ml. at end of log. phase	Population per ml. at 96 hours
Control	4.37	58,000	164,000
Minus riboflavin	5.21	19,000	67,000
Control	4.57	32,000	90,000
Minus pantothen	4.60	34,500	41,000
Control	4.32	45,500	152,000
Minus biotin	5.01	15,000	96,000
Control	4.17	49,000	170,000
Minus nicotinamide	8.40	7,500	79,000

the sixth serial transplant was possible for all series except that lacking exogenous folic acid.

In order to gain quantitative information regarding the stimulatory effect that was apparent in the tube cultures, growth flasks were inoculated from third transplant tubes and the growth followed by frequent sampling. In the case of pyridoxine, however, the flask cultures were omitted, as the Factor II preparation necessarily used was relatively inactive and the growth was erratic, even when pyridoxine was

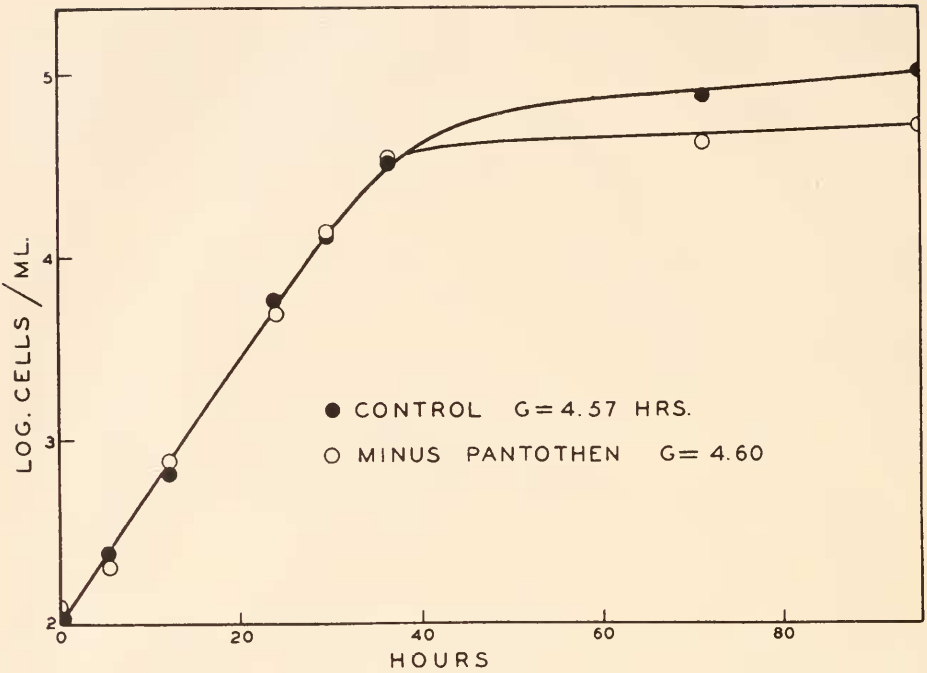

FIGURE 2. Effect of the omission of pantothenic acid. Factor II preparation used with 8L531L. Average of two experiments.

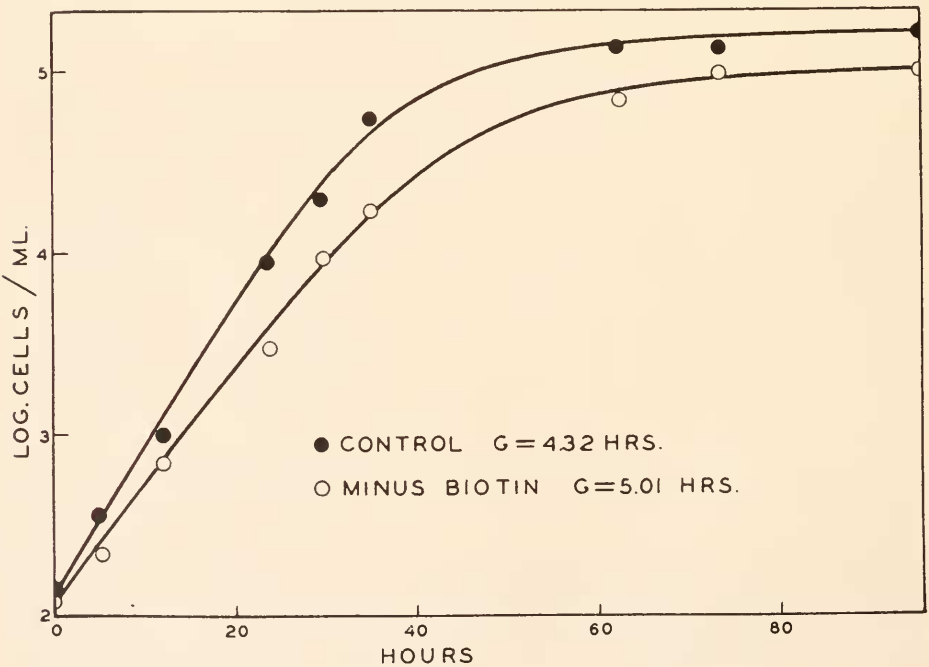

FIGURE 3. Effect of the omission of biotin. Factor II preparation used was 8L5C1. Average of two separate experiments.

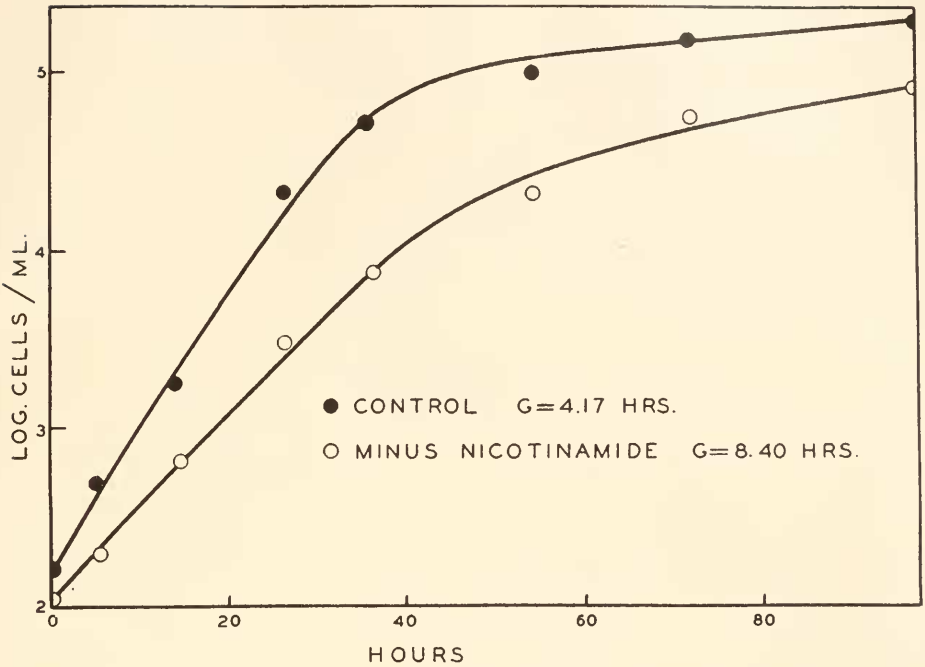

FIGURE 4. Effect of the omission of nicotinamide. Factor II preparation used was 8L5C2. Average of three separate experiments.

present. While qualitative data are lacking for the pyridixine series, nevertheless we can say from the serial tube transplants that this vitamin appears to be only stimulatory for *T. geleii* W.

The omission of riboflavin from the medium resulted in slower growth during the exponential period. Thus the generation time was raised from 4.47 hours in the control flasks to 5.21 hours. The maximum yields were reduced to less than half of those in the control flasks (Fig. 1; Table III).

The ciliates appear to synthesize pantothen at a rate which equals the demands for rapid growth, as judged by the almost identical growth rates in the pantothen-containing and the pantothen-free media (Fig. 2; Table III). In all cases, however, the maximum yield was significantly lower in the pantothen-free cultures.

A comparison of the growth curves, generation times and yields for biotin-free and riboflavin-free media (Figs. 1, 3; Table III) shows remarkable similarity. The rate of synthesis of biotin by the ciliates appears to be low, indicating the stimulatory status of this vitamin. We possess added data on biotin substantiating its non-essential nature for *T. geleii* W. Early in this series of investigations the effect of raw egg white and avidin concentrates were studied as a means of determining whether or not the ciliate required biotin. Egg white was taken aseptically and added to tubes containing 5 ml. of one per cent proteose-peptone, each tube receiving 0.1 ml. According to Eakin, Snell, and Williams (1941), this amount of egg white is enough to inactivate 0.05 micrograms of biotin. The analysis of proteose-peptone made by Stokes, Gunness, and Foster (1944) shows that one gram contains

0.2 micrograms of biotin, hence our tubes each contained 0.01 micrograms of the vitamin. The amount of raw egg white used, therefore, was enough to inactivate five times more biotin than was present. Indefinitely transplantable growth occurred in the proteose-peptone plus egg white. Likewise, the use of avidin concentrates in quantities far in excess of that needed to inactivate all of the biotin present, produced similar results. In this case the avidin was allowed to act on the proteose-peptone, the peptone removed as the diffusate in dialysis, the peptone being used as the medium. Similar results were obtained with proteose-peptone treated with H_2O_2 in a manner similar to that described by Garnjobst, Tatum, and Taylor (1943). While it is clear that biotin is not required by *T. geleii* W this vitamin is stimulatory.

TABLE IV

Assay Data (L. casei) Before and After the Growth of T. Geleii W

Additions for assay (1:10)	Factor II preparation									
	8L531		8L531H		8L5C1		8L5C2		8L531L	
	Plus riboflavin	Minus riboflavin	Plus pantothen	Minus pantothen	Plus biotin	Minus biotin	Plus nicotinamide	Minus nicotinamide	Plus pyridoxine	Minus pyridoxine
Before inoculation	4.78	0.07	3.90	0.17	3.92	0.08	3.88	0.00	3.61	0.16
After 72 hr. ciliate growth. Medium plus cells	4.60	1.18	3.94	1.71	3.90	1.56	3.80	0.21	3.48	0.22
Supernatant of 72 hr. ciliate culture	4.82	0.06	4.13	0.10	3.86	0.10	3.71	0.16	3.52	0.11
Washed ciliates from 72 hr. culture	4.80	1.07	3.94	1.64	3.91	1.05	3.75	0.25	3.61	0.15

Figures represent ml. of 0.05 N acid per culture (2 ml.). All figures corrected for uninoculated blanks and for carry-over growth.

While *T. geleii* W can be transplanted indefinitely in the absence of exogenous nictotinamide this vitamin (or nicotinic acid) is an active stimulant. The generation time is doubled when the ciliate is grown in niacin-free medium as compared to that in the nicotinamide-containing control (Fig. 4; Table III). While the population density at 96 hours is less than one-half that of the control (which is similar to the cases of riboflavin, biotin, and pantothen), the population at the end of the logarithmic phase is extremely low (approximately 7000/ml.).

It was of interest to determine whether or not *T. geleii* W would synthesize amounts of the vitamins which could be detected with the assay methods used. Accordingly the five types of media used above were set up for serial transplants and an aliquot of each was assayed with *L. casei*. After the ciliates had grown for 72 hours in the third transplants, assays were again made for the various vitamins. These assays were of three different types. One was on the whole medium (medium plus cells); one, on the supernatant fluid following centrifugation after chilling (Kidder, Stuart, McGann and Dewey, 1945), and the third was on washed cells equivalent to the concentrations found in the whole medium. The samples to be tested were added to the *L. casei* base medium and sterilized by auto-

claving. The results of these experiments are given in Table IV. Appreciable amounts of riboflavin, pantothen, and biotin are synthesized by the ciliates. Increases in amounts of niacin are so small that they probably lack significance and there appears to be no increase in pyridoxine. It must be remembered, however, that the growth in the niacin-free medium is less at 72 hours than in the riboflavin-, pantothen-, or biotin-free media, while the maximum population reached in the pyridoxine-free medium never exceeded 20,000 ciliates per ml. The amounts of the vitamins detected represent minimums, as no attempt was made to release any which may have been bound (except by autoclaving). It is to be noted that all vitamins which were synthesized remained in the cells. This was also found to be true in the case of the biosynthesis of thiamine by *T. geleii* W (Kidder and Dewey, 1942).

Discussion

Due to the various treatments necessary for the removal of vitamins none of the Factor II preparations used in this study produced as high yields as had been previously obtained (Kidder and Dewey, 1945d; Kidder, 1945). While the riboflavin-free preparation was essentially the same as had been used for the study of purines and pyrimidines and of folic acid, variations in potency of Factor II activity were evident. This is due almost entirely to the degree of adsorption on the activated charcoal. Slight variations of temperature appear to effect the degree to which Factor II is lost, so that a critical balance is found between the complete removal of the vitamins and the loss of Factor II activity. In this study the emphasis was placed on the vitamin removal at a sacrifice of yield.

The findings of Hall and Cosgrove (1944) on the importance of riboflavin for their strain of *Tetrahymena geleii* does not seem inconsistent with the present observations. They state that heat—and alkali-treated casein did not support growth unless supplemented with thiamine, and even then poorly. The addition of riboflavin together with the thiamine, however, permitted as good growth as did the casein medium before heating. There can be no doubt as to the stimulatory effect of riboflavin, and it is altogether possible that it may function as a detoxifying agent as well. The detoxifying action of thiamine has been suggested previously in this connection (Kidder and Dewey, 1944).

In addition to the vitamins which have already been investigated for *T. geleii* W there remain at least three of the commonly recognized ones about which little is known. These are *p*-aminobenzoic acid, inositol and choline. As yet we have not had the opportunity to test for the last two, but preliminary work has been started on the first. The commonly employed technique of adding sulfonamides to the medium has indicated that this ciliate requires excessive amounts of the inhibitor to effect growth. The inhibition to growth at these high levels is not completely reversed with *p*-aminobenzoic acid, and the evidence indicates that purines are also involved. This study awaits completion and will be reported at a later date, but it appears that *T. geleii* W may be independent of an exogenous supply of *p*-aminobenzoic acid.

The only other protozoan of animal nature about which there appears to be critical data regarding the requirements of the vitamins studied here is *Colpoda duodenaria* (Tatum, Garnjobst, and Taylor, 1942; Garnjobst, Tatum and Taylor, 1943).

Colpoda requires large amounts of thiamine, pantothen, riboflavin, nicotinamide, and pyridoxine. It does not require *p*-aminobenzoic acid, biotin, or inositol, while the status of choline and folic acid is still unknown. Moreover, Colpoda was shown (Garnjobst, Tatum, and Taylor, 1943) by the *Neurospora* test of Tatum and Beadle (1942) to either release bound biotin from the bacterial "plasmoptyzate" used or to synthesize this vitamin. This biotin appeared in the medium, however, and in this way differs from the condition found with *T. geleii* W where all of the vitamins arising by biosynthesis appear to bound in the cell protoplasm.

The biochemical investigations of *Tetrahymena geleii* W which have so far been completed permit a fairly complete view of its synthetic abilities. Added carbon sources appear to be unnecessary except as they may perform a sparing action on the amino acids. Inorganic salts certainly are essential (Hall and Cosgrove, 1944; Kidder and Dewey, 1944) although the question of which elements need to be included is yet to be determined. The commonly employed inorganic salts usually accepted as being physiologically important satisfy the ciliate requirements. Nine amino acids are to be classed as indispensable for this strain (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophane, and valine) while arginine is synthesized at so low a rate that its inclusion becomes obligatory. Serine is extremely stimulatory, but its place may be taken by others of the dispensable amino acids (Kidder and Dewey, 1945c). The list of essential growth factors for this strain is not long. Purines (most effectively guanine) and pyrimidines (cytidylic acid and/or uracil) must be supplied in rather large amounts (Kidder and Dewey, 1945d), and folic acid must be present in amounts in excess of that required for most of the folic acid-requiring bacteria (Kidder, 1945). Factor II must be supplied. This substance (or substances) is still chemically undefined, but it possesses similarities to the "streptogenin" of Woolley (1941) and Sprince and Woolley (1944).

Biosynthesis of riboflavin, pantothen, and biotin can be accomplished by *T. geleii* W. Indefinitely transplantable growth results without exogenous thiamine (Kidder and Dewey, 1942; 1944; 1945b), riboflavin, pantothen, biotin, niacin, or pyridoxine. There is some evidence to indicate that *p*-aminobenzoic acid may not be essential, and the status of inositol and choline is still unknown.

For practical purposes it is always of advantage to include any substances of a stimulatory nature. The absence of any one of the stimulatory substances (thiamine, riboflavin, pantothen, biotin, niacin, pyridoxine) will become a limiting factor, decreasing the growth rate or the maximum yield or the longevity of the culture (Johnson and Baker, 1942; Hall, 1944). The stimulatory vitamins should be included in the culture medium of this ciliate when maximum growth is desired.

Summary

1. It has been possible to prepare media for the growth of *Tetrahymena geleii* W which are free of riboflavin, pantothen, biotin, niacin, and pyridoxine, as determined by the *Lactobacillus casei* test.

2. *T. geleii* W is not dependent on an exogenous source of any one of the above vitamins. Omission of any one, however, reduces the maximum yield and, with the single exception of pantothen, the growth rate.

3. Biosynthesis of appreciable amounts of riboflavin, pantothen, and biotin occurs. These vitamins are found bound in the cell protoplasm. No significant increases of pyridoxine by biosynthesis were found.

4. The five vitamins listed are not essential growth factors for *T. geleii* W but are stimulatory factors, and as such should be included in the medium for optimum growth.

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